PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Rea et al.

Serial No.: 09/666,430

Filed: September 21, 2000

For: DENDRITIC CELL ACTIVATED IN THE PRESENCE OF GLUCOCORTICOID HORMONES ARE CAPABLE OF SUPPRESSING ANTIGEN-SPECIFIC T CELL RESPONSES

Confirmation No.: 6289

Examiner: G. Ewoldt

Group Art Unit: 1644

Attorney Docket No.: 2183-4205.1US

Declaration Under 37 C.F.R. § 1.132

I, ROTHING a citizen of the Netherlands, hereby declare and state as follows:

That I received a degree in BIOLOGY from LEIDEN UNIVERSITY, in 1985; and a Doctor of Philosophy in BIOMEDICAL SCIENCES from LEIDEN UNIVERSITY, in 1991;

That I am among the joint inventors of the referenced patent application;

That I conducted (or worked directly with) the series of tests related to this Declaration;

That the enclosed summary of the tests as set forth below demonstrate DEX-treated DC can exert a potent immunoregulatory effect on Th1-immunity at two levels: directly through the suppression of proliferation and IFN γ secretion by both naive and memory-type T cells, and

indirectly through the mobilization of IL-10-secreting T cells the presence of which can also suppress proliferation and IFN γ secretion by Th1 cells. The enclosed summary of the tests as set forth below also demonstrate the practical use of alternatively activated DC for modulation of the alloimmune response and show that these can induce a prolonged skin graft survival even in a complete MHC incompatible donor-recipient combination; and

That Example 1 herein was based on Example 4 of the patent application.

I further hereby declare that the enclosed summary of the tests as set forth below correctly reflect the hereinafter described materials, methods, procedures, and results of those tests.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A: Pretreatment with DEX inhibits the phenotypic change induced by LPS triggering of DC. Immature DC were cultured for 24 hours in the absence or the presence of 10^{-6} M DEX and activated with LPS (10 μg/ml) for 48 hours. Flow cytometric analysis showing the immature D1 cell line (immature DC), the LPS triggered mature DC (LPS) and the DEX pretreated LPS matured DC (DEX-LPS).

FIG. 1B: DEX exposed or control immature DC were left in culture without further treatment or stimulated with LPS. Culture supernatants were harvested 48 hours later and IL-12 secretion was analyzed by ELISA. Data are derived from 3 representative independent experiments.

FIG. 2: Pretreatment with DEX impairs the stimulatory capacities of DCs matured with LPS. Allogeneic mixed lymphocyte culture of BALB/c splenocytes with different numbers of DEX treated immature DCs (DEX), mature DCs (LPS), DEX treated mature DCs (DEX-LPS) as compared to untreated immature DCS. The IFN-γ production (B) was measured in supernatants taken after 48 hours and the proliferative response (A) was analyzed at 66 hours. Data are derived from 3 representative independent experiments.

FIG. 3: *In vivo* effects of alloreactive splenocytes by intravenous (iv) injection of immature DCs and treated DCs into allogeneic mice.

A) The proliferation of different numbers of responding splenocytes after *in vitro* restimulation with C57BL/6 splenocytes is shown after no treatment or after treatment with

either immature DC, DEX treated immature DCs (DEX), mature DCs (LPS), DEX treated mature DCs (DEX-LPS).

B) shows the IFN-γ production by the responding splenocytes at 48 hours after restimulation.

Data are derived from 2 representative independent experiments.

FIG 4: *In vivo* effects of alloreactive splenocytes by subcutaneous (sc) injection of immature DCs and treated DCs into allogeneic mice.

A) The proliferation of different numbers of responding splenocytes after *in vitro* restimulation with C57BL/6 splenocytes is shown after no treatment or after treatment with either immature DC, DEX treated immature DCs (DEX), mature DCs (LPS), DEX treated mature DCs (DEX-LPS).

B) shows the IFN-γ production by the responding splenocytes at 48 hours after restimulation.

C) shows the number of IL-10 producing cells in case the responding splenocytes were restimulated after 7 days *in vitro* stimulation with C57BL/6 splenocytes. Data are derived from 3 representative independent experiments.

FIG 5A: Graft survival of C57BL/6 (H-2^b) donor skin in untreated BALB/c (H-2^d) mice and in mice receiving 7 days before transplantation a sc injection of 1 x 10^6 mature DC (H-2^b) (LPS) or DEX treated mature DC (DEX-LPS). A significantly prolonged graft survival was found in DEX-LPS treated mice compared to mature DC treated mice ($p=0.039$).

FIG 5B: Graft survival of DBA/1 (H-2^q) donor skin in untreated BALB/c (H-2^d) mice and in mice receiving 7 days before transplantation a sc injection of 1 x 10^6 mature DC (H-2^h) (LPS) or DEX treated mature DC (DEX-LPS). No significant prolonged graft survival was found in DEX-LPS treated mice compared to mature DC treated mice ($p=0.92$).

FIG. 7: Dex-treated DC preferentially induce T cells secreting IL-10 instead of IFN γ. Non-adherent PBMC were cultured with either allogeneic CD40-triggered DC or allogeneic DEX-treated CD40-triggered DC. Supernatants were harvested at day 5 and used for measurement of IFN γ and IL-10 by ELISA.

FIG 8: T cells pretreated with modulated DC (DEX-DC) inhibit, in a dose-dependent fashion, the proliferation and cytokine production of alloreactive T cells. Alloreactive T cell

cultures were separately generated through primary stimulation of n n-adherent PBMC (donor X) during 10 days with either mature DC or DEX-DC (donor Y), after which viable T cells were isolated and counted. The resulting T cell cultures are designated as T-allo and T-dex. Subsequently, a secondary stimulation of T-allo cells was performed in the presence of mature DC (Donor X) as well as in the presence of titered amounts of T-dex. As a control, secondary stimulation was performed by mixing in titered amounts of T-allo cells. (A) Proliferation was determined after 48 hours of culture by addition of ^{3}H-thymidine for the final 16 hours. (B) Supernatants, harvested from the cultures before addition of ^{3}H-thyxnidine, were used for measurement of IFN-γ production. A representative result of three independent experiments is shown.

EXAMPLE 1

DEX-treated CD40-triggered DC are capable of suppressing Th1-type immunity

Further examination of the T cell responses induced in an allogeneic MLR by CD40-triggered DC versus DEX-treated DC (FIG. 5 of pending application) learned that induction by Dex-treated DC did not merely alter the magnitude of the alloreactive T cell response, but profoundly affected the cytokine production by the T cells stimulated. Whereas the cytokine profile of T cells induced by CD40-triggered DC primarily featured the Th1-type cytokine IFN γ, that of T cells induced by DEX-treated DC was dominated by the immunoregulatory cytokine IL-10 (FIG. 7 attached hereto). This observation prompted us to test whether the T cells induced by DEX-treated DC could themselves exert immunoregulatory properties towards T cell proliferative capacity and IFN γ secretion. Indeed, DEX-DC educated T cells, when mixed in with secondary allogeneic MLR cultures in the presence of CD4-triggered DC, were capable of strongly inhibiting the proliferation and IFN γ production by already primed alloreactive T cells.

Taken together our data demonstrate that DEX-treated DC can exert a potent immunoregulatory effect on Th1-immunity at two levels: directly through the suppression of proliferation and IFN γ secretion by both naive and memory-type T cells, and indirectly through the mobilization of IL-10-secreting T cells the presence of which can also suppress proliferation and IFN γ secretion by Th1 cells.

EXAMPLE 2

DEX-treated activated DC suppress anti-transplant immunity in vivo

Treatment of immature DC with an activating trigger in the presence of a glucocorticoid hormone results in DC maturation through an alternative maturation pathway. We demonstrate in a mouse transplantation model that such alternatively matured DC can successfully be exploited for the induction of donor-specific transplantation tolerance *in vivo*.

Materials and Methods

Mice

Female BALB/c (H-2^d), C57BL/6 (B6; H-2^b) and CBA/Ca (H-2^k) mice were obtained from IFFA Credo (Paris, France). B6.C-$H2^{bml}$/ByJ (class I K^b mutant phenotype) were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). Mice were maintained under specific pathogen-free conditions and used at 6-10 weeks of age.

Cell lines

D1 cell line, a long-term growth-factor dependent immature splenic DC line derived from B6 mice, was cultured as described (Winzler et al, 1997, Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures, *J. Exp. Med.* 185: 317). Both floating and adherent D1 cells (detached using 2 mM EDTA) were collected and used.

Treatment of DC

The D1 cells were pretreated with dexamethasone (DEX) 10^{-6} M for 24 hrs, after which LPS or nothing was added to the culture for another 48 hours. D1 treated with LPS only (for 48 hours) were also used. Both DEX and LPS (of *E. coli* (Serotype 026:B6)) were purchased from Sigma-Aldrich. After treatment, supernatants were analyzed for the presence of IL-10 and /or IL-12.

Antibodies and cell surface immuno-fluorescence

The following Abs were purchased from PharMingen: FITC-coupled anti CD86 (B7.2), PE-coupled anti CD80 (B7.1), PB-coupled anti-CD40 and PB coupled anti-I-$A^{b/d}$ (M5/114,

MHC class II). Staining was carried out at 4°C for 30 min. Stained cells were analyzed using a FACScan® flow cytometer equipped with CellQuest software (Becton Dickinson).

Cytokine analysis

Harvested supernatants were tested for IL-12 p40/p70, IL-10 or IFN-γ content using a standard sandwich ELISA. Coating Ab: rat anti-mouse IL-12 p40/p70 mAb (clone C15.6, PharMingen), rat anti-mouse IL-10 mAb (clone JES5-2A5, PharMingen) or rat anti-mouse IFN-γ mAb (clone R4-6A2, PharMingen). Detection Ab: biotinylated rat anti-mouse IL-12 p40/p70 mAb (clone C17.8, PharMingen), biotinylated rat anti-mouse IL-10 mAb (clone SXC-1, PharMingen) or biotinylated rat anti-mouse IFN-γ mAb (clone XMG1.2, PharMingen). Streptavidin-HRP and ABTS (Sigma-Aldrich) were used as enzyme and substrate, respectively. OD405 was read by an ELISAreader (Wallac, Turku, Finland).

Proliferation assays

To study alloreactivity, splenocytes (0.5 or 1 x 10^5 cells/well) of BALB/c mice were cocultured with irradiated (30 Gy, two-fold dilutions from 2 x 10^4 cells/well) D1 cells or splenocytes (30 Gy, 1 x 10^5 cells/well). Immature DC pretreated with DEX (DEX), DEX-pretreated immature DC subsequently activated with LPS (DEX-LPS) and LPS matured DC (LPS) were used as APC in the stimulation assays. The cells were plated out in U-bottom 96-well plates (Costar, Cambridge, MA, USA) in Iscove's (IMDM, BioWhittaker) containing 8% heat-inactivated Fetal Calf Serum (Greiner, Alphen, The Netherlands), 100 IU/ml penicillin, 2mM L-glutamin and 20 μM 2-ME. Supernatant was harvested after 48 hours and stored at 20°C. Wells were pulsed with 1 μCi ^{3}H-thymidine (Amersham International, Amersham, UK) and the cultures harvested onto glass fiber filters 18 hours later. Proliferation was measured as ^{3}H-thymidine incorporation by liquid scintillation spectroscopy using a betaplate (Wallac, Turku, Finland).

In vivo treatment with modulated DC analyzed by in vitro alloreactivity

After washing, 10^6 D1 cells were injected intravenously (iv) or subcutaneously (sc) in BALB/c mice in PBS with 0.5% BSA. After 7 days, spleen cells were used for detection of

alloreactive cellular responses (proliferation and cytokine analysis) by *in vitro* stimulation with splenocytes from syngeneic mice (BALB/c), from donor mice (C57BL/6) or from third party mice (CBA/Ca).

Elispot analysis

For Elispot analysis 1 x 10^6 splenocytes (from *in vivo* treated mice) were incubated with 1 x 10^6 C57BL/6 splenocytes in a 24-well plate (Costar) in Iscove's containing 8% heat-inactivated Fetal Calf Serum, 100 IU/ml penicillin, 2mM L-glutamin and 20 µM 2-ME days. The cells were harvested and incubated (at either 1 or 2 x 10^5 cells/well) with irradiated (3000 rad) splenocytes (1 x 10^5 cells/well) from C57BL/6, medium or con A controls during 24 hours in a plate (MAHA S45 10, Millipore) that was precoated with 5 µg/ml antibody (IFN-γ: R4-6A2, IL-10: JES5-2A5). Next, the wells were washed and the detection antibody was added at 0.3 µg/ml (IFN-γ: XMG1.2-biotin, IL-10: SXC-1-biotin) and incubated for 2 hours at room temperature. After another washing step, the conjugate (extravidin alkalin phospatase, Sigma E2636) was added and incubated for 1 hour at room temperature. After washing, the substrate was added and incubated for 10 minutes at room temperature, after which the reaction was stopped with tap water. Analysis of spots was performed by using a BioReader 3000 Pro (BioSys, Karben, Germany).

In vivo treatment with modulated DC analyzed by skin transplantation

After washing, 10^6 D1 cells were injected iv or sc in BALB/c mice in PBS with 0.5% BSA. After 7 days, mice were transplanted on the tail with skin grafts derived from the tail from donor mice.

The skin grafts were protected with a glass pipe of 4.5 cm long, which was kept on the tail for 7 days. Beside this protection, little irritation (and therefore inflammation) was observed because of the fact that the mice were kept on individual basis in cages with a high tech artificial bedding (Omega-Dri) instead of normal sawdust. Graft survival was followed by daily visual inspection. Scoring was performed by comparing with syngeneic grafts and was based on redness, crust-forming and the presence of hairs. The grafts were scored as rejected when they were fully necrotic or fallen off. Statistical analysis was performed using the log rank test.

RESULTS

Characteristics of alternatively activated dendritic cells (phenotype and cytokine production)

A typical FACS profile of immature DC and the influence of DEX treatment and LPS triggering on these DCs can be seen in FIG. 1A. DC matured with LPS showed significant up-regulation of CD86, CD40 and MHC class II (middle panel), when compared to immature DC, whereas DC pretreated with DEX and subsequently matured with LPS (DEX-LPS) did not show up-regulation of CD86 and only marginal up-regulation of CD40 and even a lowered expression of MHC class II (lower panel). We investigated whether DEX affected the production of the pro-inflammatory cytokine IL-12. As shown in FIG. 1B, LPS triggering of immature DC strongly induced IL-12 (p40/p70) secretion. Combined treatment with DEX and LPS resulted in a strongly reduced (7-fold) IL-12 production compared to LPS treatment alone, whereas DEX treatment only also resulted in a dramatically reduced IL-12 production.

Impaired stimulating capacity of alternatively activated DC

The reduced IL-12 production by DEX treated LPS triggered DC (DEX-LPS) prompted us to assess the T cell stimulatory capacity of these DC. As shown in FIG. 2A, proliferation of BALB/c ($H\text{-}2^d$) splenocytes in a primary MLR response to stimulation with B6 derived ($H\text{-}2^b$) DEX-LPS DC was strongly reduced (and similar to the allogeneic response to untreated immature B6 DC). Similar striking differences of the allogeneic (major and minor histocompatibility antigens mismatched) response were observed when IFN-γ production of the BALB/c splenocytes in response to the various DC used as stimulator cells was measured (FIG. 2B). Besides DEX-LPS DC also the DEX treated immature DC (DEX) induced strongly reduced alloreactive responses as measured by IFN-γ production. Therefore, these results show that mature DC pretreated with DEX have an impaired stimulating capacity.

In vivo reactivity induced by classical versus "alternatively activated" DC

To study the modulation of allo-specific immunity of these DC *in vivo*, these *in vitro* pretreated cells were injected via 2 different routes, either iv or sc. Spleen cells were harvested at different times after injection and restimulated with allogeneic splenocytes *in vitro*. Spleen

cells from mice injected iv with mature DC (LPS), exhibited a high proliferative allogeneic response which was significantly higher than that of untreated control mice or of mice treated with immature DC, DEX treated immature DC (DEX) or DEX-LPS DC (FIG. 3A). Analysis of the production of IFN-γ by the splenocytes of mice injected with the different DC revealed that mice injected iv with the DEX DC showed a similar IFN-γ production as the mice injected with DEX-LPS DC. This response was slightly higher compared to untreated control mice but significantly lower compared to mice injected with untreated immature DC or with LPS DC (FIG. 3B). The proliferative responses of spleen cells from mice injected sc with DEX-LPS DC exhibited a low proliferative allogeneic response which was similar to that of untreated controls and to that of DEX DC (FIG. 4A). The allogeneic IFN-γ response after the DEX-LPS DC treatment was slightly higher or comparable to that of untreated controls, but significantly reduced when compared to mature DC treatment, whereas the DEX DC induced a response similar to mice injected with untreated immature DC (FIG. 4B). The number of IFN-γ producing cells as measured by ELISPOT analysis was 4 times lower after treatment with DEX-LPS DC than after treatment with the DEX DC but comparable to that of untreated controls (data not shown). When the splenocytes were *in vitro* stimulated with C57BL/6 alloantigens for 6 days and restimulated with either conA or C57BL/6 splenocytes, the ELISPOT analysis showed an increase in the number of IL-10 producing cells when compared to untreated or to LPS-DC treated mice (FIG. 4C).

The third-party reactivity was not altered in the DC treated mice compared to untreated mice, indicating that the treatment with alternatively activated $H\text{-}2^b$ DC was specific for the $H\text{-}2^b$ alloantigens. These experiments demonstrate that DEX-LPS DC induce an alloimmune response, which, based on the *in vitro* parameters tested, showed both quantitative and qualitative differences compared to the alloimmune response found after injection with mature DCs.

Prolonged skin allograft survival after injection with alternatively activated DC

Subsequently, we analyzed the *in vivo* "modulatory" potential of the DEX-LPS DC in a fully allogeneic skin graft model. BALB/c mice were injected sc with either LPS DC or DEX-LPS DC or left untreated. One week after treatment these mice were transplanted with a skin

graft derived from the tail of a donor C57BL/6 mouse. The skins derived from C57BL/6 mice were rejected by the mice injected with LPS DC with a median survival time of 14 days which is not significantly different from the survival in untreated mice (MST 16 days, FIG. 5A). However, when mice were transplanted after injection with DEX-LPS DC, a significantly prolonged allograft survival was found (MST 34 days p=0.039). A similar significant prolongation was observed in 2 other independent experiments using BALB/c mice as responding strain (p=0.023 and p=0.009) and in another study using BM1 mice as responder strain (p=0.008, data not shown).

The prolonged skin graft survival after treatment with alternatively activated H-2^b DC was specific for the H-2^b alloantigens as mice injected with DEX-LPS DC rejected skin grafts from DBA/1 mice (H-2^q) in the same time (MST 14 days, FIG. 5B) as control mice (MST 14 days, untreated or LPS DC treated mice p=0.90, p=0.92 resp). These results show that the DEX-LPS DC are capable of inducing a specific prolongation of complete MHC incompatible skin allograft survival.

DISCUSSION

The present study shows that addition of a glucocorticoid hormone to immature DC results in a decreased proliferative response and a decrease in IFN-γ production by BALB/c splenocytes stimulated by these DCs. In addition we demonstrate that *in vivo* treatment with DEX pretreated mature DC decreased the allogeneic Th1 response as shown by a reduced IFN-γ production *in vitro* and a reduction in number of IFN-γ producing effector cells when the response was compared to mice pretreated with mature DC. This was the case both after sc or iv injection of the DEX pretreated DCs, but even more after *in vivo* treatment with the alternatively activated (DEX-LPS) DC. Pretreatment of recipients with these DC leads to a significantly prolonged skin graft survival.

In conclusion, our studies confirm and extend the practical use of alternatively activated DC for modulation of the alloimmune response and show that these can induce a prolonged skin graft survival even in a complete MHC incompatible donor-recipient combination.

I hereby declare that all statements made herein of my own knowledge are true and that

all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the U.S. Code and that such willful false statements may jeopardize the validity of the patent.

RIENK OFFRINGA
[name]

20 AUGUST, 2003
Date

Residence: LEIDEN, The Netherlands
Post Office Address: STIGTSTRAAT 63, The Netherlands

Date: April 2, 2003
KWP/((Initials))

\\Srv-am1\Octrooien-am\Chemie\Secretariaat\Mill\Doorn\nieuw\P 49219 US 00 (dexamethason)\02042003 Declaration .doc